Structure and Function of Proton-Translocating Adenosine Triphosphatase (F_0F_1): Biochemical and Molecular Biological Approaches

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INTRODUCTION

As early as 1924, Albert J. Kluyver discussed the unity and diversity of metabolism of microbes (128). He pointed out that there must be some underlying thermodynamic unity between microbes, although different organisms can use different energy sources in the medium. After decades of critical experiments, we know now that ATP plays a central role in energy transduction and that the main principle of the catalysis and the essential features of the molecular organization for ATP synthesis in membranes have been conserved throughout evolution. Most ATP is synthesized by oxidative and photophosphorylation in mitochondria (or bacterial cytoplasmic membranes) and chloroplasts, respectively. The overall mechanisms of ATP synthesis in these organelles are now well explained by the chemiosmotic theory. The theory, proposed by Peter Mitchell, formulates that ATP is synthesized by the ATPase complex utilizing an electrochemical gradient of protons (153). The respiratory or photoelectron transfer chain in the energy-transducing membranes forms an electrochemical gradient of protons through the unidirectional movement of protons across membranes. The electrochemical gradient of protons consists of two components, ΔpH (transmembrane pH gradient) and $\Delta\psi$ (electrical potential). The ATPase complex, a different membrane entity from the electron transfer chain, phosphorylates ADP to form ATP coupled with a reverse flow of protons. The complex can work reversibly and forms an electrochemical gradient of protons coupled with hydrolysis of ATP. Thus, the ATPase has been named ATP synthase (or synthetase). However, the ATPase complex in certain organisms functions only for the formation of an electrochemical gradient of protons through hydrolysis of ATP (89). In some cases, this enzyme from bacteria has been called Mg^{2+}, Ca^{2+}-ATPase, but we think this name is not appropriate, since it leads to confusion of the enzyme with ATPases of divalent cation transport.

The ATPase complex has been called H^+-translocating ATPase or H^+-ATPase because of its functional coupling to proton movement and

ATP synthesis or hydrolysis (154). The complex consists of two main portions, F_1 (peripheral membrane portion) and F_0 (integral membrane portion) (Table 1). The catalytic portion, F_1, is an extrinsic membrane protein and consists of five different subunits, α, β, γ, δ, and ϵ. F_0 is apparently a transmembrane complex, mediating proton translocation between two compartments of the organelle. Proton passage through F_0 is regulated by the F_1 portion. The F_0 portion has been studied less extensively than the F_1 portion, but the subunits of F_0 (a, b, c) from *Escherichia coli* have been unambiguously identified in biochemical and genetic studies (61, 77, 88, 117).

The ATPase complex was first studied in mitochondria and chloroplasts and later in bacteria. However, once the universal nature of the complex became apparent, it was realized that studies on bacterial ATPase are promising for understanding the structure and catalytic mechanism of F_0F_1. Extremely stable F_0F_1 was isolated from a thermophilic bacterium and was reconstituted into liposomes (108). One advantage of using bacteria, especially *E. coli*, is that genetic techniques can be applied. Since the first mutant of *E. coli* with a defect in F_0F_1 was isolated (26), genes for each subunit have been identified (79, 80). The gene cluster was cloned and found to be located on a specific DNA segment (44, 119, 122, 235). DNA sequencing of the genes coding for the entire complex is now complete, and the primary sequences of all of

the subunits of F_0F_1 from *E. coli* are known (76, 77, 112, 114–118, 138, 161, 190). Analysis of the sequence gives an insight into the structure-function relationship of the enzyme. Another obvious advantage of using bacteria is that we can easily isolate mutants containing subunits in which a single amino acid residue is altered. Altered functional residues of certain subunits have been determined or shown to be located in certain domains of polypeptide chains, and now we are not so far from being in a position to discuss the function of F_0F_1 in molecular terms. Needless to say, the bacteria also provide an ideal system for study of the assembly or physiological regulation of the complex by genetic methods.

As discussed above, structural and functional studies on F_0F_1 from plant, animal, and bacterial sources have been carried out. Thus, it is impossible to cover all of the publications on this subject comprehensively in a limited space. In this article we concentrate on bacterial F_0F_1, mainly that of *E. coli*, and try to stress the significance of combined biochemical and molecular biological approaches in bacteria. We also include recent results on F_0F_1 from other sources, such as results on the kinetics of the beef heart enzyme, as they are pertinent for understanding the entire complex. For fields that we discuss only briefly or do not mention, the reader should refer to the excellent review articles available mainly on F_0F_1 from chloroplasts (8, 149, 150), mitochondria (36, 175, 180), and bacterial membranes (43, 50, 58, 72–74, 79, 80). The mechanism of phosphorylation has been discussed extensively (13, 38, 214). Detailed reviews on the F_0 portion are also available (57, 204).

SYNTHESIS AND HYDROLYSIS OF ATP COUPLED TO $\Delta\bar{\mu}_{H^+}$

The overall reaction in oxidative and photophosphorylation consists of two steps, formation of $\Delta\bar{\mu}_{H^+}$ by electron transfer reactions and synthesis of ATP utilizing this electrochemical potential of protons. A series of experiments initiated by Jagendorf and Uribe (107) established that an artificially imposed electrochemical gradient of protons could drive synthesis of ATP in chloroplasts (250), chromatophores (133), or submitochondrial particles (227), suggesting that ATP synthesis by F_0F_1 is coupled to $\Delta\bar{\mu}_{H^+}$. Later Kagawa and co-workers showed that F_1F_0 only is responsible for $\Delta\bar{\mu}_{H^+}$-driven synthesis of ATP (181, 219). They purified F_0F_1 with eight subunits from a thermophilic bacterium, PS3, and incorporated it into phospholipid liposomes. ATP was synthesized when ΔpH was applied to the liposomes in the presence of K^+ and valinomycin (219) or an electric field was

TABLE 1. Nomenclatures of F_0F_1 subunits and their structural genes[a]

Subunit			Structural gene (*E. coli*)		
(i)	(ii)	(iii)	(i)	(ii)	(iii)
F_1 α		1	*uncA*	*papA*	*atpA*
β		2	*uncD*	*papB*	*atpD*
γ		3	*uncG*	*papC*	*atpG*
δ		5	*uncH*	*papE*	*atpH*
ϵ		8	*uncC*	*papG*	*atpC*
F_0 a	χ (24K)	6	*uncB*	*papD*	*atpB*
b	ψ (19K)		*uncF*	*papF*	*atpF*
c	Ω (8K)	9	*uncE*	*papH*	*atpE*
14K			*uncI*	*papI*	*atpI*

[a] Subunit nomenclature (i) has been widely used for F_0F_1. Fillingame proposed the named χ, ψ, and Ω for the F_0 subunits previously named as 24K, 19K, and 8K, respectively (ii) (58). Nomenclature (iii) has been used mainly for the yeast enzyme (230). Subunits 4 and 7 are not listed above, because we do not know the corresponding bacterial subunits. Three nomenclatures for structural genes are listed above: *unc*, uncoupled oxidative phosphorylation (26); *pap*, proton-translocating ATPase protein (118); *atp*, ATPase-translocating protons (88).

applied in a voltage-pulse experiment (181). Liposomes with F_0F_1 of eight subunits from either E. coli (157) or PS3 (165) were also capable of forming $\Delta\bar{\mu}_{H^+}$ upon hydrolysis of ATP. These experiments suggest that only eight subunits of F_0F_1, when properly assembled, are sufficient for synthesis and hydrolysis of ATP coupled to $\Delta\bar{\mu}_{H^+}$. However, other proteins, including those with regulatory functions, may be required for mitochondrial or chloroplast systems. These proteins include F_6 (3), factor B (101), and ATPase inhibitor (174). Primary structures of ATPase inhibitors from yeast (146) and beef heart (64) have been obtained. However, no protein of homologous structure could be found from the DNA sequence of the F_0F_1 gene of E. coli, as discussed below. Preparations from mitochondria (188, 215), chloroplasts (178), or chromatophores (9, 166) could be reconstituted into liposomes in which they carried out the ATP-phosphoric acid (P_i) exchange reaction or ATP synthesis.

As pointed out by Maloney (144), the physiological driving force for ATP synthesis differs in different organelles. This difference may be due to differences in the buffering capacity of the cytoplasm or medium and in the ion permeability of the membranes. Thus, mitochondria utilize $\Delta\psi$ predominantly, whereas chloroplasts use ΔpH; bacteria are intermediate and can use either type of energy depending on the conditions of the medium. This capacity to use either $\Delta\psi$ or ΔpH may be advantageous in adaption of bacteria to different environments. Maloney and Schattschneider (145) also pointed out from studies on Streptococcus lactis that the total $\Delta\bar{\mu}_{H^+}$ is the driving force of ATP synthesis, not the absolute value of $\Delta\psi$ or ΔpH. Furthermore, identical rates of ATP synthesis were observed when an equivalent ΔpH or $\Delta\psi$ was imposed. A model of energy coupling that accommodates the quantitative interconversion of ΔpH and $\Delta\psi$ may be required for understanding the system, including the "proton well" (which converts $\Delta\psi$ into the equivalent as $-Z\Delta pH$ at the interface between F_0F_1) proposed by Mitchell (153, 154). In connection with this, Gibson and collaborators recently isolated an interesting mutant (80). The F_0F_1 of this mutant is apparently unable to synthesize ATP coupled with respiration dependent on NADH, but is able to synthesize ATP with $\Delta\bar{\mu}_{H^+}$ in an artificial system. This mutant seems to be somehow defective in energy coupling, possibly in the interconversion of $\Delta\psi$ and ΔpH. Detailed studies with bacterial F_0F_1 may be useful in understanding the interconversion of the two components of $\Delta\bar{\mu}_{H^+}$. The H^+/ATP stoichiometry can be calculated from $\Delta\bar{\mu}_{H^+}$ and the phosphorylation potential and is three for chloroplasts and E. coli (124) and two or three

for mitochondria. A list of measurements is available, and the stoichiometry is discussed in a previous review (57).

F_0F_1 functions as either an ATP synthase or an ATPase. In mitochondria and chloroplasts, F_0F_1 functions in ATP synthesis. In aerobic bacteria, F_0F_1 functions in both ATP synthesis and $\Delta\bar{\mu}_{H^+}$ formation (89, 185). Thus, aerobic bacteria can generate $\Delta\bar{\mu}_{H^+}$ by hydrolysis of ATP even in the presence of enough respiratory inhibitor to abolish respiration (10). In anaerobic bacteria such as Streptococcus faecalis which have no electron transfer chain, F_0F_1 behaves as a true H^+-translocating ATPase, extruding H^+ to maintain $\Delta\bar{\mu}_{H^+}$ (89, 144). The $\Delta\bar{\mu}_{H^+}$ formed by respiration or ATP hydrolysis directly supports many energy-requiring reactions in bacteria, such as active transport, the transhydrogenase reaction, and flagellar movement (89). It is also required to maintain the pH and ionic composition of the bacterial cytoplasm. In this regard, it is noteworthy that Kobayashi et al. showed that F_0F_1 is responsible for regulation of cytoplasmic pH in S. faecalis (129). Under normal conditions, the cytoplasmic pH is regulated in this organism by extrusion of protons mediated by F_0F_1 and by influx of K^+ by a specific transport system to compensate for $\Delta\psi$.

GENETICS of F_1F_0

unc Mutants and Mapping of the Mutations

F_0F_1 mutants have been isolated, and the mutations have been mapped at approximately 83 min (between the asn and glmS genes) on the linkage map of E. coli (26, 43, 152) (Fig. 1). They were selected by (i) loss of growth on unfermentable carbon sources, such as succinate (but positive growth on glucose or glycerol) (43); (ii) resistance of neomycin (123, 182); or (iii) resistance to aurovertin (192) or dicyclohexylcarbodiimide (DCCD) (56, 204). Mutants selected by (i) and (ii) could not synthesize ATP via oxidative phosphorylation with succinate as the respiratory substrate, although their respiration itself was normal. Thus, they showed uncoupled oxidative phosphorylation. Butlin et al. first isolated a mutant of this type and called it an unc strain (26). unc mutants were also isolated from neomycin-resistant colonies, because this antibiotic is accumulated in cells by an energy-dependent process and inhibits translation. It is evident from the mode of action of neomycin that a neomycin-resistant mutant is not always an unc mutant. DCCD and aurovertin inhibit growth of wild-type strains, thus providing a third procedure for selection. As these inhibitors are known to bind to F_0F_1 as discussed below, the F_0F_1 from these mutants showed altered interaction with DCCD (204) or aurovertin (192). However,

these mutants were apparently not defective in oxidative phosphorylation (56, 192, 204). They were selected by growth on an unfermentable carbon source (succinate) in the presence of these inhibitors. In this condition, cells with defective oxidative phosphorylation could not multiply. In all of the F_0F_1 mutants isolated so far, the mutation is mapped in the same region as in the first unc mutant. The genetic locus that codes for F_0F_1 was termed unc. These studies have made it possible to isolate a series of mutants by localized mutagenesis with P1 phage transduction (43, 103, 113, 119, 228). Three groups of workers including ourselves have proposed changing the nomenclature to that directly related to the gene product (Table 1): bcf for bacterial coupling factor (185), atp for ATPase-translocating protons (88), and pap for proton-translocating ATPase subunit protein (118). Our proposal was based on the fact that all structural genes were recently assigned to components of F_1F_0 and the idea that the genetic nomenclature should be closely related to the actual gene product. However, we use the term unc gene in this review to avoid possible confusion.

Organization of the unc Operon

Gibson and co-workers (81) isolated a series of unc mutants and transferred their mutant
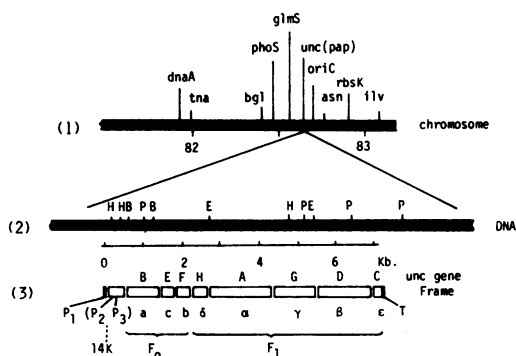

FIG. 1. E. coli linkage map and organization of the gene cluster for proton-translocating ATPase. (1) E. coli linkage map for the region around unc (pap or atp). The positions of genes and genetic symbols are as described by Miki et al. (151, 152). (2) Physical map of the unc (pap or atp) gene cluster. (3) Approximate positions for coding frames for subunits of F_1 and F_0, indicated by open boxes. A reading frame for a protein of 14,000 daltons (14K) was found from the DNA sequence. A promoter sequence, P_1, was found to be active by in vitro transcription (116). Two promoter-like sequences, P_2 and P_3, were also found (117). A typical terminator region (T) was found to hybridize with a promoter sequence (P_1) (114). The scale (kilobase pairs [kb]) and cleavage sites for endonucleases are also shown: H, HindIII; E, EcoRI; B, BamHI; P, PstI. See text for details.

alleles of F′ plasmids covering the ilv-unc region. These F′ plasmids were transferred to haploid unc strains, resulting in partial diploid strains containing two mutant unc alleles. It was concluded that mutations on the F′ plasmid and on the chromosome were located in different genes, if the diploid strains could grow on succinate or were not uncoupled. Seven genes, uncA, -B, -C, -D, -E, -F, and -G, have been defined by such complementation assays (43, 79, 80). The order of the transcription of the gene cluster was studied by using polar mutants induced by insertion of bacteriophage Mu, and it was concluded that the seven genes form one operon and are transcribed in the order uncB(E,F), -A, -G, -D, -C (42–45). The structural genes uncE and -F were ordered unambiguously by DNA sequencing as discussed below. The promoter of the operon is upstream of the uncB gene and proximal to the asn gene. The organization of the gene cluster was finally determined by DNA sequencing of the entire operon. Gene products were assigned to subunits by analysis of the mutant complex on two-dimensional gel (210, 211), in vitro reconstitution of ATPase with mutant F_1 and wild-type subunit (46, 121), and in vitro protein synthesis (44, 86).

Different approaches have been used to define the gene cluster. When we started genetic studies, it was not clear whether all structural genes of the subunits of F_0F_1 were located in the 83-min region of the bacterial chromosome or whether some of the subunits were coded in other regions. In fact, only five genes were known in 1979 (43), although we were sure that the entire complex was coded by more than five genes. First, we showed that all of the structural genes for F_0F_1 are located between glmS and oriC on the E. coli chromosome (119). Miki et al (151) and von Meyenburg et al. (236) isolated defective transducing λ phages carrying segments of the chromosome from around the uncA gene in studies on the origin of replication of E. coli. These phages were temperature inducible, but defective in lysing cells. Thus, it seemed possible to prepare membranes after induction and assay increase in the amount of F_0F_1, if these phages carry the entire set of genes for F_0F_1. We found that all of the structural genes were carried by λ asn5, because we showed that subunits of F_1 and the activity of functional DCCD-sensitive ATPase increased in the membranes with increase in number of phages after induction (119). In collaboration with Fillingame and co-workers, we demonstrated that each of eight polypeptides found in the preparation of F_0F_1 was overproduced during thermoinduction of the transducing phages (63). Furthermore, with λ asn5 DNA as a template, in vitro transcription-translation experiments by Brusilow et

al. gave eight polypeptides corresponding to those in the F_0F_1 polypeptides (24). These results suggest that all of the genes for F_0F_1 are carried by the transducing phage λ $asn5$ and that F_0F_1 may have eight subunits (three for F_0 and five for F_1).

These studies facilitated identification of the gene cluster in a defined portion of the physical map. Initial studies on the *E. coli* DNA carried by three different transducing phages had indicated that all of the genes for F_0F_1 were located within a DNA segment that could be digested to units of 1.7, 3.0, and 5.7 megadaltons with *Eco*RI endonuclease (119). We isolated several transducing phages and plasmids carrying various lengths of DNA segments of the *unc* operon (122) and tested them for genetic complementation with our stock of independent mutants and a few previously described strains. Analysis of DNAs from transducing phages and plasmids with restriction endonucleases suggested that all of the structural genes for F_0F_1 are located within a DNA segment of approximately 4.5 megadaltons (7.0 kilobase pairs) containing two *Eco*RI sites (122) (Fig. 1). The size of the DNA is approximately the same as that calculated from the sum of the molecular weights of the eight polypeptides of F_0F_1. The physical map of the gene cluster has been obtained. Mutations of all of the mutants tested could be mapped on this DNA segment, including those of *uncA401*, *uncB402*, *uncD11*, and mutations of the *c* subunit (122, 226). We obtained a recombinant plasmid, pMCR533, carrying a *Hind*III fragment of λ $asn5$ and found that this DNA fragment contained about 70% of the gene cluster. Von Meyenburg and co-workers mapped the *unc* gene in the same region and located the genes for subunits approximately by analysis of the proteins expressed by various transducing phages and recombinant plasmids (88, 235). Downie et al. also cloned the *Hind*III fragment and located the *uncB*, *-F*, *-E*, *-A*, and *-G* genes on this fragment from results on in vitro protein synthesis (42).

DNA Sequence of the Genes Coding for F_0F_1

As discussed above, we mapped the genes for F_0F_1 within a defined DNA segment of approximately 7 kilobase pairs which is carried by the transducing phage λ $asn5$ (122). We have determined the complete nucleotide sequence of this DNA segment (112, 114–118, 138). Nielsen et al. (161) independently determined the DNA sequence of the genes for the *a*, *c*, *b*, δ, and α (amino-terminal portion) subunits, using transducing phage λ $asn105$, which carries genes for eight subunits of F_0F_1. Walker and co-workers also determined the DNA sequence of the entire

operon, using λ $asn5$ (76, 77, 190). The DNA sequences reported by the three groups were essentially the same.

From these findings, the organization of the gene cluster was finally established. The genes for the three F_0 components proximal to the promoter precede the genes for subunits of F_1 (Fig. 1). The significance of this organization in gene expression is unknown. The order of the structural genes is *uncB* (*a*), *uncE* (*c*), *uncF* (*b*), *uncH* (δ), *uncA* (α), *uncG* (γ), *uncD* (β), *uncC* (ε). Genes were located from the amino-terminal sequence (*b*, δ, α, γ, β, and ε subunits) and complete amino acid sequence (*c* subunit). The amino acid compositions calculated from the sequence were also in good agreement with those obtained by chemical analysis of the protein. The Shine-Dalgarno sequence (217), a sequence complementary to the 3' end of 16S RNA, was observed within a reasonable distance upstream of the initiation codon of each reading frame. The coding sequence for subunit *a* was estimated from genetic studies. Recent determination of the amino terminus of this subunit (unpublished data cited in reference 93) indicated that the start position suggested earlier was correct. The above gene order was confirmed by in vitro protein synthesis, using an extensive set of plasmids, which carried parts of the gene cluster (86). This sequence of the DNA segment gave us unambiguous information on the subunit composition. It is especially noteworthy that the *a*, *b*, and *c* subunits were determined as authentic F_0 subunits from the DNA sequence. Upstream of the *uncB* gene, we found an open reading frame capable of coding for a protein of 130 amino acid residues. The designation *uncI* (*atpI* or *papI*) may be appropriate for this frame (237), although another group named it gene 1 (77). The putative protein has been called 14K protein or 14KD polypeptide. From its amino acid sequence, the frame seems to be a highly hydrophobic protein (25% polar residues), which is a characteristic of membrane components. However, we cannot conclude that 14K protein is a subunit of F_0F_1 because we have no evidence for its presence in purified F_0F_1: no protein of corresponding molecular weight was detected in purified active F_0F_1 by electrophoresis (61, 66). The 14K protein has been suggested to regulate the biogenesis and assembly of F_0F_1 (77, 117). Recent results by von Meyenburg et al. suggest that the *uncI* gene is not essential for the biosynthesis or activity of F_0F_1 (237). They isolated Tn*10* insertion mutations within the *uncI* reading frame. Their insertion mutations led to a three- to eightfold decrease in expression of genes downstream of the frame, as detected by decreased syntheses of the *c*, α, and β subunits. However, the defect of

these mutations could not be complemented by a high-copy-number plasmid, pFH350, carrying the *uncI* gene, although the defect was complemented by pBJ917 carrying all of the structural genes except *uncI*. Thus, mutations are *cis* dominant, suggesting that the 14K protein of the *uncI* gene product, even if it exists, is not essential for biosynthesis and activity of F_0F_1.

A typical promoter sequence was found in the region upstream of the open reading frame for the 14K protein (77, 116). This sequence was identified as an active promoter for the operon by in vitro transcription and footprint analysis (116). Promoter-like sequences were found in the reading frame for the 14K protein and are indicated as P_2 and P_3 in Fig. 1 (77, 116). Both have sequences similar to the Pribnow box, but P_2 does not have a typical sequence for the -35 region. The significance of these two promoter sequences is so far unknown. Recently, von Meyenburg et al. (237) analyzed Tn*10* insertions into the frame for 14K protein and found two different groups for the expression of the genes located downstream. The insertions closest to *uncB* resulted in an 80 to 90% decrease in the *c* subunit, whereas those 150 to 200 base pairs upstream resulted in a 60 to 70% decrease. They suggested from these effects that this operon has two weak promoters together with a major promoter. These two weak promoter sites possibly coincide with P_2 and P_3 found in the DNA sequence. Kanazawa et al. pointed out sequences similar to a Pribnow box in the intercistronic region between the genes for the *a* and *c* and *c* and *b* subunits (117), although the significance of these sequences for gene expression is unknown. No typical promoter-like sequence (Pribnow box and -35 region) was found in the intercistronic region.

A sequence characteristic for termination of transcription was found in the region downstream of the *uncC* gene (114, 190). We found a complementary structure between the promoter and terminator regions of the putative mRNA (114). A similar complementary structure was also found in the tryptophan operon, but not in *recA* (114).

The F_0F_1's in eucaryotic cells are formed by subunits coded by nuclear and organellar genomes, although all subunits are coded by a single operon in *E. coli*, as discussed above. The entire nucleotide sequences of human (6) and mouse (11) mitochondrial DNA have been determined recently. The yeast mitochondrial DNA contains genes for subunit 9 (DCCD-binding protein) and subunit 6 (corresponding to *E. coli* subunits *c* and *a*, respectively [Table 1]) as different transcriptional units (91, 139, 140). On the other hand, mammalian mitochondrial DNA contains only the gene for subunit 6 (6, 11). Genes for the chloroplast β and ϵ subunits (130,

257) and the *c* subunit (100) were also sequenced. The genes for β and ϵ are cotranscribed into a single mRNA with overlapping of four bases between the stop codon of the β gene and initiation codon of the ϵ gene (257). A gene for the α subunit was mapped in a region about 40 kilobase pairs away from a gene cluster for β and ϵ (257). In vitro translation experiments suggested that subunit I (probably corresponding to subunit *a*) of spinach chloroplast F_0 was coded by chloroplast DNA (160). Mitochondrial or chloroplast gene products assemble with the cytoplasmically synthesized subunits coded by nuclear genomes (232). Available sequence data indicate that primary translation products of F_0 subunits coded by *E. coli* or the organellar genome do not have a leader sequence, suggesting no processing during assembly into membrane. In contrast, *c* subunit coded by the nuclear genome of *Neurospora crassa* has a precursor form: a nuclear gene (cDNA) for subunit *c* was cloned and the amino acid sequence of the preprotein (66 polar residues attached to the amino terminus of the *c* subunit) was determined (234). A nuclear gene for the β subunit of *Saccharomyces cerevisiae* was also cloned and a partial DNA sequence was determined (M. K. Douglas, personal communication).

Synthesis and Assembly of F_0F_1

F_0F_1's differ in stoichiometry of their eight subunits, as discussed below (α_3, β_3, γ, δ, ϵ, *a*, b_2, c_{3-15}). One question is, what is the regulatory mechanism to achieve this stoichiometry? Genetic evidence from *E. coli* indicated the presence of a single polycistronic mRNA for all eight subunits of this operon (79, 80). DNA sequencing studies showed that there is only a single copy of each gene within the operon and also suggested the presence of a single mRNA for the operon, as no typical promoter-like sequences were found in the noncoding region or structural genes of the subunits. Therefore, it is not possible that genes for multicopy subunits are transcribed more efficiently than those for single-copy subunits. Possible regulation at the translational level was suggested from analyses of the usage of synonymous codons and the secondary structure of mRNA. Based on the content of isoaccepting tRNA and the nature of codon-anticodon interaction, Ikemura (105, 106) predicted the order of preference among synonymous codons and named the most preferred codon the "optimal codon." *E. coli* genes for abundant protein use optimal codons selectively, whereas other genes use optimal and nonoptimal codons to almost equal degrees. Kanazawa et al. (114) applied this prediction to genes for F_0F_1 subunits and found that the frequency of usage of the optimal codon (usage of optimal

codons/that of total codons) was clearly higher in the multicopy subunit (0.85) than in the single-copy subunit (0.7 to 0.6), suggesting that the amount of subunits is determined, at least to some extent, by the frequency of codon usage in each gene. Simoni and co-workers (25) observed that a plasmid carrying eight genes directed the syntheses of the eight subunits in unequal amounts in vitro and in minicells. They attributed this unequal synthesis to the secondary structure of the mRNA. They found loop and stem structures immediately preceding and including initiation codons for the *uncF* (*b*), -*H* (δ), and -*G* (γ) genes and proposed that these structures are responsible for the low expression of these genes. However, there may be more stem-loop structures in other regions of the operon, and it may be pertinent to study their role before making any conclusion on the specific function of the above structure. Although codons and stem-loop structures may regulate translation, they cannot explain the mechanism of generation of a complex with an exact subunit ratio. To explain this, we should also study other possibilities such as that of autogenous regulation of synthesis of ribosomal proteins (251) or selective degradation of unassembled polypeptides. Although not discussed here, there must be more detailed and complex regulatory mechanisms for the synthesis of F_0F_1 in mitochondria of chloroplasts, because their subunits are coded by both organellar and nuclear genomes.

Recently, Cox et al. (34) proposed a unique scheme for the assembly of F_0F_1 into the cytoplasmic membrane of *E. coli* from analyses of a series of mutants. The most pertinent part of their model is that F_1 subunits α and β are assembled with an *a* subunit before formation of functional F_0 with *b* and *c* subunits. In this way an open proton pathway of F_0 (*a*, *b*, *c*) is not formed during assembly. However, this model may not be true for the following reasons. (i) Five subunits of F_1 can be assembled in vitro without the F_0 component, as discussed below (49). (ii) Decker et al. (41) showed that all three F_0 subunits could be synthesized and inserted into membranes or liposomes in vitro, and this insertion was independent of the synthesis of each peptide and of F_1 polypeptides. (iii) Friedl et al. (68) showed that the *a* and *b* subunits could be assembled into membranes in vitro without synthesis of other subunits. Thus, more studies are required to establish the mechanism of assembly of this complex membrane enzyme.

F_1, CATALYTIC PORTION OF THE ATPase

Preparation and Structure of F_1

F_1 could easily be released from membranes, in most cases simply by washing the membranes

with dilute buffer, and it has been purified by conventional procedures from a variety of sources. Purified F_1 has ATPase activity, although its specific activity varies somewhat depending on its source. The F_1's purified from various sources have striking similarities in structure and enzymological properties. Recently, Muñoz comprehensively reviewed bacterial F_1's and the methods used for their solubilization and preparation (156). The F_1 obtained from most sources is composed of five different subunits, α, β, γ, δ, and ε, in order of decreasing molecular weight estimated by electrophoresis. However, minor subunits in different preparations may not be equivalent, although the same nomenclature has been applied to them. Sequencing studies indicated that mitochondrial δ is homologous to *E. coli* ε, and the bacterial δ is related to the oligomycin sensitivity-conferring protein of mitochondria (244). The subunit stoichiometry, $α_3β_3γδε$, has been established for F_1's from thermophilic bacterium PS3 (109) and *E. coli* (16, 58, 62, 191), although discrepant results have been obtained on other F_1's (72). However, it is improbable that different F_1's have different subunit stoichiometries, because the subunits of F_1's, especially the β subunit, show a high degree of homology, as discussed below.

The F_1 from the thermophilic bacterium PS3 (108), TF_1, and that from mitochondria (4, 5, 168, 221) have been crystalized. Image reconstruction of the two-dimensional crystal of TF_1 indicated a pseudohexagonal structure (242). The three-dimensional crystal of rat liver mitochondria diffracts up to a resolution of 0.35 nm on X-ray diffraction (4, 5). Amzel et al. observed a distorted hexagonal or octahedral arrangement (4). Mitochondrial F_1 appears to be formed by two equivalent portions, each of which consists of three equal masses, A, B, and C, with overall dimensions of 11 by 12 by 8 nm and a molecular weight of 380,000. Assuming $α_3β_3γδε$-type stoichiometry, Amzel et al. proposed that the A and B masses contain the α and β subunits, respectively, or each mass contains domains of α and β, whereas the C region contains α or β with low-molecular-weight subunit γ, δ, or ε. Their model is extremely interesting, because it predicts that α and β exist in two different environments or possibly different conformations. They also pointed out the possibility that the α or β subunit may have different functions in different environments. The molecular weight and dimensions of *E. coli* F_1 were also deduced from small-angle X-ray (172) and neutron (194) scattering. The values obtained by the two methods were as follows: molecular weight, 358,000 and 340,000; radius of gyration, 4.19 and 4.6 nm; maximal dimension, 12 and 13 nm, respectively. These values for the molecular weight of *E. coli* F_1

(EF$_1$) are slightly lower than that calculated from the amino acid sequence (382,113) assuming $\alpha_3\beta_3\gamma$-type stoichiometry. Difficulties of determining the exact value of the complex in solution may be due to dissociation of the minor subunits during the experiment. Models of F$_1$ attached to F$_0$ through the δ and ϵ subunits have been proposed mainly from studies of interaction of subunits and reconstitution of F$_1$ (72, 108) (Fig. 2A). These models are consistent with the electron microscopic picture of negatively stained membranes: spheres of 9 nm attached to the bilayer through a portion called the stalk. Todd and Douglas (230, 231) proposed a different model for yeast F$_1$ from studies of cross-linking and proteolysis (Fig. 2B and C). In their new version, the α subunit oriented at the membrane is the associated portion of F$_1$ (Fig. 2B). A similar model was also proposed for EF$_1$ from genetic studies (34, 79). The results of Todd and Douglas are also compatible with a model of different orientation (Fig. 2D): in this model α oriented in the cytoplasmic side of the complex. More studies seem to be required to establish the exact structure of F$_1$. Models of F$_0$F$_1$ and F$_0$ are discussed below.

Mechanisms of Synthesis and Hydrolysis of ATP on F$_1$

There has been rapid progress in studies on the synthesis and hydrolysis of ATP with F$_0$F$_1$. Detailed discussion of this subject can be found in a recent review (214), but here the recent progress is summarized because we hope to visualize finally the structure-function relationship of F$_0$F$_1$, although most studies on the mechanism were carried out on beef heart F$_1$. As discussed above, the F$_1$ portion forms the catalytic domain of the entire complex and solubilized F$_1$ still retains ATPase activity. A striking difference of F$_0$F$_1$ ATPase from ion transport ATPase is that no covalent phosphoenzyme intermediate of F$_1$ has been found, whereas such intermediates of other ATPases have been found (155). Recent evidence suggests that F$_1$ is probably not a phosphoenzyme. Webb et al. (247) synthesized adenosine 5'-(3-thiotriphosphate) labeled with ^{18}O in the γ position and analyzed
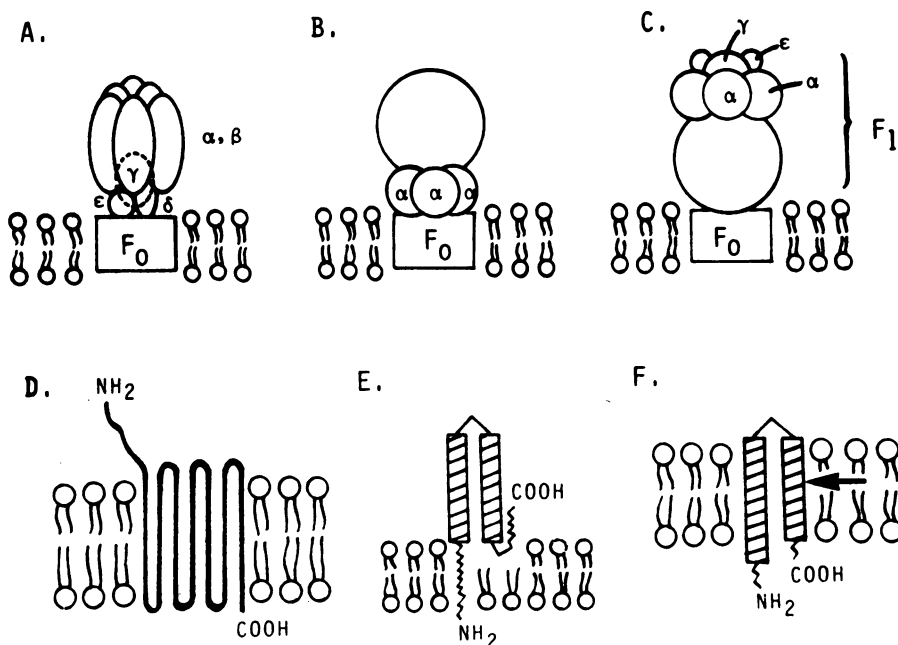

FIG. 2. Models of F$_0$F$_1$. Models are not drawn to scale. (A) Conceptual model of F$_0$F$_1$ of *E. coli* mainly based on reconstitution experiments (49, 72, 108). (B and C) Models of yeast F$_0$F$_1$ compatible with data obtained by cross-linking and protease treatment (231). (D) Model of *E. coli* subunit *a* drawn from the estimation of hydrophobic segments (60, 196, 203). The secondary structure was not estimated here because of high hydrophobicity of the proteins. (E) Model of the *E. coli b* subunit extruding from a phospholipid bilayer. Hatched boxes indicate α-helical domain. In this model a portion embedded in the membrane was drawn as a β sheet, but this portion may be also α helical as predicted by Senior and Wise (214). (F) Model of the *E. coli c* subunit modified from that previously published (204, 214). Hatched boxes indicate α-helical domain. The arrow indicates the approximate position of the DCCD-binding residue.

its hydrolysis with F_1 in $^{17}O-H_2O$ from the ^{31}P-nuclear magnetic resonance spectrum. They suggested that there is a direct in-line transfer of phosphoric acid residue between ADP and water and thus that there is no phosphoenzyme intermediate. Recently, Boyer and colleagues showed that F_1 catalyzes an exchange between water-oxygen and oxygen of phosphate during steady-state hydrolysis of ATP at low concentration (30, 104). However, this exchange was not observed with a high concentration of ATP. These results suggest that ATP hydrolysis at the catalytic site is reversible and that cooperative interactions occur between different sites.

One mole of F_1 usually contains 3 mol of non-exchangeable nucleotide, which apparently does not participate in catalysis (38, 40) and is stable in actively metabolizing *E. coli* (143). The fate of bound nucleotides in EF_1 (2 mol of ATP and 1 mol of ADP) (142) was studied in vivo in label-chase experiments (143). Two-thirds of the bound ATP prelabeled with ^{32}P was retained over generations, whereas bound ADP was chased rapidly. Thus, at least 2 mol of nucleotide was stable metabolically in this F_1. The roles of these nucleotides have been suggested to be in maintaining the complex subunit structure of F_1 (143, 214). Cross and Nalin showed that beef heart F_1 already containing 3 mol of such nucleotides could still bind an additional 3 mol of adenyl-5'-yl-imidodiphosphate (AMP-PNP) in a strongly cooperative fashion (40). The K_d values of the first site and of the second and third sites were 18 nM and about 1 μM, respectively. F_1 with only 1 mol of AMP-PNP bound at the first site could not dissociate the nucleotides, but it released AMP-PNP with the binding of ADP possibly at the second site. Other evidence is also consistent with the presence of three exchangeable nucleotide sites in F_1 (38).

Grubmeyer and Penefsky have shown the cooperativity of ATP hydrolysis, using beef heart F_1 and 2',3'-O-(2,4,6-trinitrophenyl)-ATP (84, 85). This analog bound to the first catalytic site and was hydrolyzed very slowly. However, the apparent binding efficiency in the first site decreased by a factor of 10^5 to 10^6, and thus the rate of hydrolysis was accelerated 20-fold, when the second site was occupied with the substrate. They proposed that F_1 undergoes a conformational change associated with conversion of the second site to the tight site after release of ADP + P_i from the first site. Cooperativity among the three sites during hydrolysis of [γ-^{32}P]ATP was also suggested (39, 83). The ATP bound tightly at the first site and was hydrolyzed only slowly. However, upon binding of unlabeled ATP at the second site, [γ-^{32}P]ATP at the first site was hydrolyzed without a lag and at a rate close to the maximal velocity. The initial velocity of F_1

was measured with a wide range of ATP concentrations. Gresser et al. showed that the substrate-velocity data are consistent with the predicted curve for three interacting sites, but not for independent catalytic sites of the Michaelis-Menten type (82). Cooperativity among the various nucleotide binding sites of the F_1's of beef heart (148) and *E. coli* (147) was also shown, using dansyl-ATP [2'-(5-dimethyl-aminonaphthalene-1-sulfonyl)amino-2'-deoxy ATP]. Two moles of this analog bound to 1 mol of beef heart F_1 with a K_d of 0.44 μM. Binding of the analog and formation of the enzyme product complex were rapid processes. Release of dansyl-ADP + P_i from F_1 was a slow process, but it was markedly accelerated by the addition of ATP of high concentration. From these results, Matsuoka et al. suggested that ATP bound to a tight catalytic site and that its hydrolysis was stimulated by ATP bound to a low-affinity regulatory site (148). They did not discuss the possibility that the catalytic and regulatory sites alternated during catalysis, but their observation is consistent with other results.

Hutton and Boyer suggested the reversibility of the catalytic site of F_1 (104). Grubmeyer and Penefsky (85) analyzed the hydrolysis of ATP at the first tight site and found that the equilibrium constant for ATP $\rightleftharpoons$ ADP + P_i was 0.5. These results suggest that this equilibrium can occur without free energy. Feldman and Sigman (55) obtained an equilibrium constant of 2 and actually observed synthesis of enzyme-bound ATP (0.25 mol/mol of chloroplast F_1 [CF_1]) from P_i in the medium and nucleotide in CF_1. Boyer and colleagues could not detect [^{32}P]ATP from $^{32}P_i$ and beef heart F_1 (30, 104). They attributed this lack of ATP formation to the slow rate of the $P_i \rightleftharpoons HOH$ exchange reaction (1/1,000 of the rate with submitochondrial particles). Sakamoto and Tonomura recently overcame this difficulty by using dimethyl sulfoxide and observed synthesis of bound ATP (0.13 mol/mol of F_1) with beef heart F_1 in buffer containing 30% dimethyl sulfoxide (189). The role of dimethyl sulfoxide in their system is still unknown; possibly it changes the affinity of ADP or P_i during catalysis. These results suggest that energy is not required for the formation of ATP in the catalytic site of F_1 and that the sites for ATP hydrolysis in isolated F_1 are the same as those for ATP synthesis with F_0F_1 in oxidative or photophosphorylation. Furthermore, these results support the "binding change mechanism" of phosphorylation by F_0F_1 (12). The mechanism proposed has three essential features: (i) cooperativity between multiple catalytic sites, (ii) formation of enzyme-bound ATP without energy input, and (iii) energy-linked changes in binding of ATP and ADP + P_i. Many factors must be examined before this

mechanism can be finally established; in particular, the conformational change induced by site-site cooperativity must be shown to be related to that induced by proton pumping. In this regard Hammes proposed a model in which ion transport ATPases exist in two possible conformations and the pumping of ions is associated with the transition from one conformation to the other (87). The energy-linked conformation change has been summarized (12). Mitchell proposed a mechanism of phosphorylation in which a pair of protons moving through F_0 directly participate in the catalysis (154, 155). In his speculative model ADP, P_i, and $2H^+$ (properly transported through F_0) form an intermediate in the catalytic site of F_1 and dissociation of the OH_2^+ group with an electron forms ATP and water. This mechanism is not consistent with results discussed above, especially those showing that isolated F_1 synthesizes ATP without a proton gradient.

Primary Structures of α, β, and γ Subunits

The primary sequence of each subunit from *E. coli* was determined from the DNA sequence (76, 77, 114–118, 138, 161, 190). Information on each subunit, such as its number of residues, molecular weight, and polarity (29), is summarized in Table 2. The α, β, and γ subunits have polarity similar to those of soluble proteins: polarities of α, β, and γ are 43, 44, and 45% (Table 2), respectively. Capaldi and Vanderkooi (29) showed that 85% of 205 soluble proteins had a value of 47 ± 6%. The amino acid sequence of the α subunit is partially homologous with that of the β subunit, suggesting that these two

polypeptides have the same ancestral origin, as discussed previously (114, 246). Walker and coworkers determined the primary sequence of β from bovine F_1 by the protein chemical procedure and found that 70% of the residues are homologous with those in β from *E. coli* (246). The primary sequences of the β subunits of CF_1's from spinach and *Zea* maize were determined from the nucleotide sequence of the chloroplast DNA (130, 257). Their primary sequences also show about 70% homology with that of β of *E. coli* (Table 3). Of the subunits, this subunit may have the most conserved primary sequence in different species, although similar comparisons are only possible now for the ε, *a*, and *c* subunits. Homologies in these subunits are also summarized in Table 3.

The α and β subunits both have binding sites for ATP and ADP, as revealed by studies on chemical modification and isolated subunits, although it was difficult to identify specific residues of the sites. However, information on sequence homology suggests the possible residues that form binding sites, because similar residues may be involved in the catalytic or binding sites of different enzymes for which the substrates or effectors are ATP or ADP. Thus, we think that information on sequence homology will be extremely useful for identifying nucleotide binding sites, using a biochemical or genetic approach, in future. We found that a portion of the sequence of α is similar to that of alanyl and tyrosyl tRNA synthetase of *E. coli*, nitrogenase reductase of cyanobacteria, and the β subunit of RNA polymerase of *E. coli* (112). It is striking that these proteins including α have a

TABLE 2. Properties of subunits of F_0F_1 of *E. coli*[a]

Subunit	Mol wt	No. of amino acid residues	Polarity	Estimated secondary structure (%)		% α-helical content (from circular dichroism)	
				α helix	β sheet	*E. coli*	PS3
F_1							
α	55,259	513	0.43	46.4	19.1		31
β	50,117	459	0.44	27.2	13.7		34
γ	31,414	287	0.46	35.0	15.0		49
δ	19,303	177	0.44	61.0	19.2	~75	65
ε	14,914	138	0.44	43.4	12.3	~40	33
F_0							
a	30,275	271	0.32	23.6	21.7		
b	17,244	156	0.48	76.9	8.3		
c	8,246	79	0.23	50.6	31.6	60–80	
14K	14,087	130	0.25				

[a] Numbers of residues and molecular weights are for the primary structure determined from the DNA sequence. Data on the polarity (calculated as described previously [29]) and secondary structure are cited from Kanazawa and Futai (112). The α-helical contents of the δ (223), ε (224), and *c* (98, 204) subunits of *E. coli* were determined previously. The α-helix contents of subunits of thermophilic bacterium PS3 are cited from Yoshida et al. (256).

TABLE 3. Homology in the primary structure of F_0F_1 subunits[a]

Source	Homology (%)			
	F_1		F_0	
	β	ε	a	c
E. coli	100	100	100	100
Beef	72[b]			18[c]
Maize	66[d]	23[d]		
Spinach	67[e]	26[e]		
S. cerevisiae	70[f]		11[g]	16[c]
Mouse			3[h]	
Thermophilic bacterium PS3			38[c]	

[a] Amino acid sequences of corresponding subunits from E. coli and other sources were aligned to obtain maximal homology. Homology was expressed by percent homologous residues, taking the total residues of each E. coli subunit as 100%. Primary structures of E. coli subunits were from Kanazawa and Futai (112).
[b] From Walker et al. (246).
[c] From Sebald and Hoppe (204).
[d] From Krebbers et al. (130).
[e] From Zurawski et al. (257).
[f] Partial sequence kindly provided by M. G. Douglas (University of Texas, San Antonio).
[g] From Macino and Tzagoloff (140).
[h] From Bibb et al. (11).

common sequence of Glu-Arg-Gly-Leu-Ala. Walker et al. found significant homology of α with Ca^{2+} ATPase, ATP/ADP translocator, adenylate kinase, and phosphofructokinase (243, 246). We found that residues 150 to 156 in the sequence of β are similar to those of adenylate kinase and that residues 186 to 202 are similar to those in recA protein of E. coli (115) (Fig. 3). Homologies were also found between β and myosin (rabbit and nematode) or aspartokinase (246) (Fig. 3). Furthermore homology was found between the oncogene product (p21) and the β subunit, suggesting that p21 may have membrane-bound nucleotidase activity (78). It is noteworthy that the sequences of the β subunit that were homologous with those of other enzymes were mapped in three regions of the primary structure (Fig. 3). The secondary structures of all subunits were estimated by the method of Chou and Fassman (31) from the amino acid sequence (74, 114, 138). However, it must be noted that the estimation was carried out without consideration of interactions between subunits or phospholipid and subunits (especially those of F_0). It is known that the α subunit of EF_1 changes conformation after binding of ATP (47, 167, 169, 170, 208). Table 2
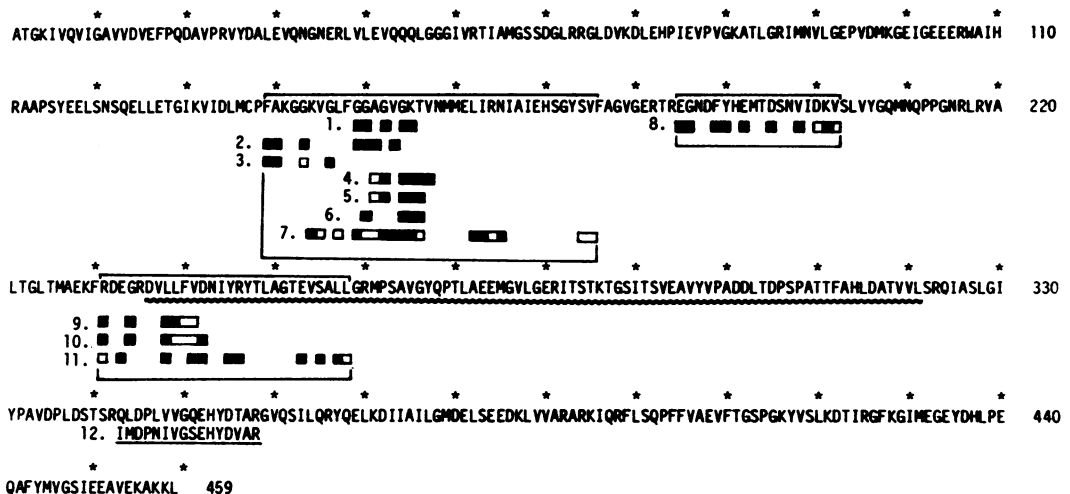

FIG. 3. Primary sequence of the β subunit of E. coli showing regions homologous with those in other enzymes. Portions of the primary structure of the β subunit homologous with those in enzymes capable of nucleotide binding are as follows: 1, residues 15 to 21 of adenylate kinase (114); 2, residues 191 to 206 of nematode myosin (243, 246); 3, residues 196 to 203 of rabbit myosin (243, 246); 4, residues 180 to 186 of rabbit myosin (243, 246); 5, residues 177 to 182 of nematode myosin (243, 246); 6, residues 66 to 73 of recA protein and residues 170 to 177 of E. coli α subunit (243, 246); 7, residues 5 to 36 of human C-bas/ras[H] p21 fragment (78); 8, residues 266 to 282 of recA protein (114); 9, residues 107 to 117 of adenylate kinase (243, 246); 10, residues 279 to 290 of ATP/ADP translocator (243, 246); 11, residues 143 to 172 of nematode myosin (and also residues 147 to 176 of rabbit myosin) (243, 246); 12, a peptide of β which bound p-fluorosulfonyl [^{14}C]benzoyl-5'-adenosine (52, 53). Y(Tyr) is a radioactive derivative of Tyr that was sulfonylated during inactivation. Identical and conserved residues are shown by solid and open bars, respectively. The portion of the subunit forming a Rossmann fold (186) was deduced from the primary structure (114) and is underlined (∿) in Fig. 5. The references cited above are to papers in which homologies are discussed.

summarizes the estimated contents of α-helices and β-sheets of the subunits. The estimated helical contents of α, β, and γ are similar to the average values for soluble proteins. Similar values were obtained for isolated subunits of TF_1 or EF_1 by circular dichroism measurement (223, 224, 256). An alternating structure of α-helix and β-sheet (Rossmann fold) is known to bind nucleotide in dehydrogenase, kinases, elongation factor Tu, and other nucleotide binding enzymes (186). A similar domain was observed in the β-subunit around residues 240 to 330, which is the highly conserved region in the β subunits of different F_1's (114, 246) (Fig. 3). This folding structure includes a region that is homologous with those in other enzymes and may be related to the ATP binding site. No similar domain was found in the estimated secondary structure of the α subunit (74).

Properties of Isolated α, β, and γ Subunits

All five subunits of F_1 have been obtained only from *E. coli* (49, 70, 223, 224) and the thermophilic bacterium PS3 (110, 253, 255). Single subunits or mixtures of two subunits from either EF_1 or TF_1 had no ATPase activity, but a complex having ATPase activity could be reconstituted from the α, β, and γ subunits (70, 110), and the entire F_1 could be reconstituted from this complex and the δ and ε subunits (49, 255). Methods for isolation of the subunits and the properties and reconstitution of the subunits have been reviewed extensively (50, 72). The results discussed above are in apparent disagreement with the finding that the two-subunit complex (α + β) obtained by trypsin treatment had full ATPase activity (159). Recently, Smith and Sternweis (218) purified ATPase after extensive trypsin digestion and found that on gel electrophoresis the trypsin-treated enzyme gave α and β subunits together with two protein bands close to the tracking dye. These two low-molecular-weight bands reacted with antibodies against the γ subunit but not with antibodies against the other four subunits. Although it is unknown whether these two polypeptides were derived from different parts of the γ subunit, the results suggest that a fragment(s) of the γ subunit remained tightly bound even after extensive trypsin treatment. This fragment(s) requires further study, because it may be derived from a domain of the γ subunit that interacts with the α and β subunits in intact F_1.

ATPase activity could be reconstituted from mixtures of subunits of different F_1's, suggesting that the subunits of F_1 of different species are functionally homologous. Such experiments were first made on the subunits of TF_1 and EF_1. Three combinations showed ATPase activity (75): (i) β of TF_1 and α and γ of EF_1, (ii) α and β

from EF_1 and γ from TF_1, and (iii) α and β of TF_1 and γ of EF_1. These results suggest that subunits of the two different bacteria have similar roles and structural homologies. The ATPase reconstituted from α and β of TF_1 and γ of EF_1 was similar to TF_1 in being activated by Na_2SO_3, methanol, and sodium dodecyl sulfate, whereas other hybrids (i and ii) showed properties similar to those of EF_1 (225). These results suggest that α or β or both is required for the properties of F_1. The β and γ subunits of *Rhodospirillum rubrum* were recently isolated sequentially by successive extractions of the chromatophores with LiCl and LiBr (125, 176). Isolated β and β + γ could rebind to β-less and β- and γ-less chromatophores, respectively, with restoration of photophosphorylation activity (125). The isolated β subunit of this bacteria and α and β subunits of *E. coli* could form hybrid ATPase (data obtained in collaboration with Z. Gromet-Elhanan and D. Khananshvili, unpublished data). We also isolated the α, β, and γ subunits of F_1 from *Salmonella typhimurium* and found that any mixtures of the three major subunits from this bacterium and *E. coli* showed ATPase activity (data obtained in collaboration with S.-Y. Hsu, M. Senda, and T. Tsuchiya, manuscript in preparation). These results suggest that even at the subunit level F_1's are functionally the same in a wide range of bacteria. A functional similarity at the subunit level is also shown from immunological studies. Antibodies against *R. rubrum* β subunit inhibited ATP-linked reactions not only in chromatophores but also in chloroplasts (177). Furthermore, antibodies against β from rats, yeast, chloroplasts, and *E. coli* cross-reacted with corresponding subunits of all four F_1's tested (187). The functional and structural similarities of subunits from different origins were also suggested from the homology of the primary structures of subunits as discussed above.

The α subunit of *E. coli* had a single high-affinity site with K_d values of 0.1 and 0.9 μM for ATP and ADP, respectively (49). The isolated α shows a large conformational change upon binding to the nucleotide with increase in diffusion and sedimentation coefficients and change of other physical parameters (47, 167, 169, 170). Senda et al. detected an ATP-dependent conformational change of α, using trypsin (208). The subunit was cleaved with a small amount of trypsin (1 μg/mg of subunit) to small peptides, whereas it was cleaved to two main polypeptides of 30,000 (amino-terminal half) and 25,000 (carboxyl-terminal half) daltons in the presence of sufficient ATP to saturate the high-affinity site. These results suggest that the conformation of the subunit changed to form two trypsin-resistant domains upon binding of ATP to the high-

affinity site. Dunn obtained a dissociation rate constant of the αATP complex of 0.21 min^{-1} (47). This dissociation rate constant is about 10^6-fold lower than that of the enzyme-substrate complex in general. Dunn concluded that the site in α is not a catalytic site, assuming that the properties of the binding site of α do not change upon assembly with other subunits. On the contrary, Matsuoka et al. recently suggested that the high-affinity site in α may be a catalytic site (147). The dansyl-ATP in the high-affinity site of F_1 was hydrolyzed as discussed above, although dansyl-ATP bound to isolated α was not hydrolyzed. Both isolated α and F_1 have relatively high-affinity sites for dansyl-ATP with apparent K_d values of 0.06 and 0.23 μM, respectively, whereas no binding was detected in isolated β subunit. Assuming that the K_d for dansyl-ATP of α was altered fourfold on isolation of the subunit, they suggested that dansyl-ATP in α of F_1 was hydrolyzed. It is uncertain at present which subunit has the catalytic site, although there seems to be more evidence that it is in the β subunit or the interface between α and β subunits than in α as discussed previously (72, 213) and below. From circular dichroism analysis, Ohta et al. suggested that ADP bound to α of TF_1 at low concentration and to both the α and β subunits at higher concentration (163).

No nucleotide binding site has yet been found directly in the isolated β subunit of *E. coli*. However, binding was detected with 1-anilinonaphthalene 8-sulfonate (71), the fluorescence of which increased upon the addition of purified β; this fluorescence was quenched on addition ATP (10^{-3} to 10^{-4} M), suggesting that β has a nucleotide binding site of low affinity. The presence of one low-affinity site in β from TF_1 was suggested from circular dichroism measurements (163). The same method showed the presence of one high-affinity site in α from TF_1 (163, 164).

Interactions between different subunits have been examined by studies on isolated subunits and their reconstitution. Dunn et al. found that the amino-terminal portion of the α subunit is required for binding the δ subunit to the αβγ complex (51). This finding extended the observation of Abrams and co-workers, who showed that F_1 from *S. faecalis* could not bind to membranes after chymotrypsin treatment that resulted in reduction of the molecular weight of the α subunit (1, 2). Dunn also showed specific formation of a complex containing single copies of the γ and ε subunits (48). High-affinity specific interactions between γ and ε are also indicated by three lines of evidence: (i) the isolation of a complex of these two subunits, (ii) specific quenching of fluorescence of Trp residues in γ by ε, and (iii) inhibition by γ of the interaction of ε and the αβγ complex. Close interaction of γ

and ε has been shown by cross-linking experiments on F_1's of mitochondria (231), chloroplasts (7), and *E. coli* (19). The K_d for the γε complex was estimated to be 3 nM (48). The interactions of α (7, 230) and β (7, 19) with ε were also shown by cross-linking experiments. The models of F_1 in Fig. 4 include these subunit interactions.

Chemical Modification and Photoaffinity Labeling of α and β Subunits

We now discuss recent results on chemical modification, mainly of EF_1. Lunardi et al. (135) reported preferential labeling of the α subunit of EF_1 by 3'-O-(4-(4-azido-2-nitrophenyl) aminobutyl)ATP at a low concentration (5 μM), but almost equal labeling of both α and β by this compound at a higher concentration (75 μM). Although total inactivation was observed at a higher concentration of the analog, only 25 to 30% of the enzyme was inactivated at the low concentration, suggesting that the high-affinity site in α may not be the catalytic one. The α subunit of EF_1 was labeled with 8-azido ATP (233) or dialdehyde ATP (20) with concomitant
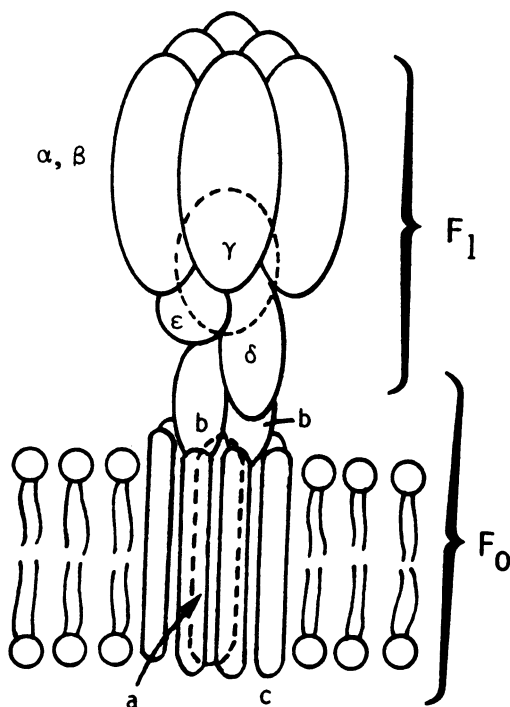

FIG. 4. Conceptual model of F_0F_1 of *E. coli*. A conceptual model of F_0F_1 was drawn based on reconstitution experiments, subunit stoichiometry, predicted secondary structures, and subunit interactions discussed in the text. This model is not drawn precisely to scale.

loss of ATPase activity. However, 8-azido ATP labeled the β subunits of *Micrococcus* sp. (197) and mitochondrial (241) F_1's. 6-((3-Carboxyl-4-nitrophenyl) thio)-9-β-D-ribofuranosyl purine 5'-triphosphate (92, 102) and 8-azido-1-N-etheno ATP (195) predominantly labeled the β subunit of *Micrococcus* sp. F_1. Esch and Allison (52, 53) used ^{14}C-labeled p-fluorosulfonylbenzoyl-5'-adenosine, which reacts with nucleophilic amino acid residues, for identification of the nucleotide binding sites of beef heart F_1. The inactivation of F_1 by this reagent was correlated with incorporation of the label into the β subunit. Only one peptide had radioactivity, and a Try residue was found to be labeled (Fig. 3). Lunardi and Vignais (137) synthesized a photoaffinity analog of AMP-PNP and incubated it with mitochondrial F_1 which had about 3 mol of tightly bound nucleotide. They observed predominant labeling of α with a low concentration of the analog, but labeling of both α and β with a higher concentration. These results seem to exclude the possibility that all tightly bound nucleotides are in α subunits. As the extent of inactivation with the analog was independent of the nature of the photolabeled subunit (α or β), they suggested that both subunits are required for the activity of F_1. As discussed above, Cross and Nalin showed that F_1 has three sets of nonexchangeable and exchangeable (catalytic) sites for AMP-PNP (40). The results with the analog suggest that one exchangeable site is in the α subunit or that three sites are formed from α and β subunits and that they have different conformations depending on the substrate concentration, as suggested from kinetic results. The latter possibility seems more probable, because different conformations could give the different labeling patterns observed. From studies with the photoaffinity label 3'-O-(4-benzoyl)-benzoyl ATP, Williams and Coleman (248) extended this view and suggested that the catalytic site of mitochondrial F_1 may be at the interface of the α and β subunits. Bruist and Hammes (23) also suggested that the dissociable nucleotide binding site may be located near the α–β subunit interface of CF_1. Although results on nucleotide binding sites are somewhat confusing, we could draw the tentative conclusion that the catalytic site may be at the interface between α and β. In this regard it is important to identify the residues labeled with the analog as in the case with p-fluorosulfonylbenzoyl adenosine. Further studies with EF_1 may be especially useful, because the primary structures of the subunits of EF_1 are known. Furthermore, a final conclusion could be drawn from comparison of the results of chemical modifications, genetic studies, and studies on isolated subunits. A photoreactive analog of phosphate (4-azido-2-nitrophenyl phosphate) has

been shown to label the β subunit, suggesting that this subunit has a phosphate binding site (132).

The effects of general protein-modifying reagents on F_1 have been studied. DCCD inactivates both TF_1 and EF_1. This reagent causes pH-dependent inhibition of soluble F_1 (179, 193): the F_1 was inhibited at a slightly acidic pH, but not at an alkaline pH, whereas membrane-bound F_1 was inhibited under both conditions. Recently Allison and co-workers identified a DCCD-labeled Glu residue of the β subunits of *E. coli* (252), thermophilic bacterium PS3 (254), and mitochondria (54). The regions in the vicinity of this Glu residue in the three F_1's are highly homologous (Fig. 5). However, it is noteworthy that although DCCD reacted with homologous residues of β from mitochondria and *E. coli*, it reacted with a different Glu residue (11 residues from the above Glu) of TF_1. It is interesting that the sequence around this particular Glu residue of *E. coli* is homologous to recA protein, which has ATPase activity (Fig. 5). The rate of inactivation of all three F_1's are reduced by the presence of Mg^{2+}. Therefore, the two Glu residues may function to bind Mg^{2+} as suggested by Allison and co-workers, or the domain including these residues may undergo an Mg^{2+}-dependent conformational change that is essential for the catalysis. These results indicate the importance of determining the residues modified by chemical reagents, rather than speculating on results obtained by modification with so-called specific reagents. However, other protein chemical studies did not reach this point. Recently, Ting and Wang (229) carried out a series of kinetic experiments on the inactivation of EF_1 in a short period with 4-chloro-7-nitro-2-oxal-1,3-diazole (NBD-Cl), DCCD, phenylglyoxal, fluorodinitrobenzene, and 2,4,6-trinitrobenzene sulfonate in the presence and absence of ADP, ATP, P_i, or Mg^{2+}. They suggested that Tyr, Lys, Asp or Glu, and Arg are functional residues at the catalytic site, as suggested for mitochondrial F_1. NBD-Cl (4-chloro-7-nitro-2-oxa-1,3-diazole) is a potent inhibitor of F_1. Most of the label of this compound was on α after a short incubation time (233), but it was on β after a long incubation time (159). Modification of F_1 with NBD-Cl caused loss of the nucleotide binding activity of EF_1 (135, 136), mitochondrial F_1 (137), and CF_1 (28); Bragg and Hou obtained spectral evidence indicating that NBD-Cl modified a Tyr residue (18). A combination of reconstitution and chemical modification studies was carried out on F_1 of *R. rubrum* (126). The β subunit modified with DCCD could not bind to β-depleted chromatophores, whereas β modified with NBD-Cl bound to the β-depleted chromatophores with restoration of both ATP synthesis and hydrolysis.
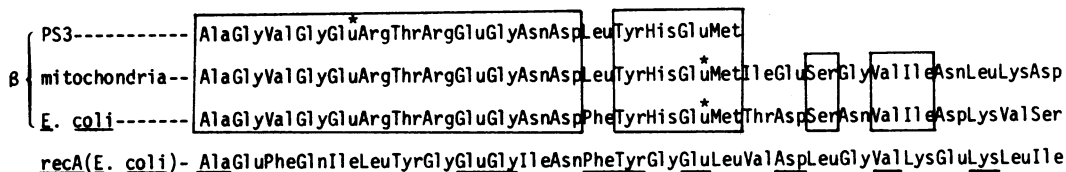
```
        ┌ PS3 ----------- │AlaGlyValGlyGluArgThrArgGluGlyAsnAsp│Leu│TyrHisGluMet│
                                                   *
      β ┤ mitochondria -- │AlaGlyValGlyGluArgThrArgGluGlyAsnAsp│Leu│TyrHisGluMet│IleGlu│Ser│Gly│ValIle│AsnLeuLysAsp
                                                                        *
        └ E. coli ------- │AlaGlyValGlyGluArgThrArgGluGlyAsnAsp│Phe│TyrHisGluMet│ThrAsp│Ser│Asn│ValIle│AspLysValSer
                                                                        *

      recA(E. coli) - AlaGluPheGlnIleLeuTyrGlyGluGlyIleAsnPheTyrGlyGluLeuValAspLeuGlyValLysGluLysLeuIle
```

FIG. 5. Amino acid sequence around the DCCD-reactive glutamic acid residues of β subunits and the region homologous with that of recA protein. Glu* indicates the N-glutamyl derivative of dicyclohexyl [^{14}C]urea. Results on the DCCD-reactive site are cited from Esch et al. (54) and Yoshida et al. (252, 254). The sequence of the recA protein is cited from Horii et al. (99).

Mutations of α, β, and γ Subunits

The role of subunits in catalysis and assembly can also be studied by using F_0F_1 with mutationally altered subunits. Genetic alterations of subunits are highly specific, and mutant subunits can be obtained in which a single amino acid residue is changed. By identifying the altered residues, it is then possible to map the essential residues in the primary structure of each subunit. However, there are difficulties in biochemical analyses of F_0F_1 mutants: genetic alteration of a single amino acid residue can change the properties of the entire complex or possibly the assembly process, making it difficult in many cases to isolate inactive F_1 complexes from mutants (43, 58, 73, 111). Thus, the inactive F_1's of only a few mutants have been prepared and characterized. A recent list of useful mutants is available (58): the most thoroughly characterized mutant is AN120 (uncA401) (26), which has defective α (46, 121, 210). Purified F_1 from this mutant had no ATPase or ATP synthesis activity, but had normal subunit composition and normal nucleotide binding activity (18, 249). This F_1 has the normal molar content of tightly bound nucleotides (141), but the nucleotide binding sites of AN120 showed different kinetics from wild-type F_1 for binding of dialdehyde ATP (21). Wise et al. (249) observed that in wild-type F_1 the fluorescence of aurovertin bound to β increased with the addition of ADP to saturate the high-affinity site, whereas this response was not observed with F_1 from AN120. This observation was essentially confirmed by other groups (21, 112). Aurovertin was shown to bind to the β subunit in studies on the isolated subunit (49) and on an aurovertin-resistant mutant (192). Thus, the most reasonable interpretation of the results is that the conformation of the binding site for aurovertin in β changes on binding of ATP to the high-affinity site. This conformational change, possibly an interaction between α and β subunits, is essential for the ATPase and is defective in AN120.

Senior et al. described five strains carrying mutations in the β subunit (uncD) (211). Only one (uncD412) of these strains formed F_1 that could be purified as a complex. The purified F_1 had a normal molecular weight and subunit structure, but only 10% of the ATPase activity of wild-type F_1 and an abnormal ratio (about 2) of Ca^{2+} ATPase to Mg^{2+} ATPase. Kanazawa et al. identified a mutant, KF11, of the β subunit (uncD11) (113). The F_1 from this mutant had about 10% of Mg^{2+}-dependent ATPase activity of the wild type, with properties similar to those of uncD412. The ratios of Ca^{2+}- and Mg^{2+}-dependent ATPase activities of the wild-type and KF11 were 3.5 and 0.8, respectively. Recently, Senior et al. reported the properties of 17 new mutants (212). Only four of them had an abnormal F_1 complex that could be purified to homogeneity. The F_1 preparations from uncD430, uncD431, and uncD484 had 1.7, 1.8, and 0.2% of the wild-type ATPase activity, and all of these strains had negligible ATP synthesis activity. Strain uncD478 had F_1 with a relatively high specific activity of ATPase (15% of normal) and of ATP synthesis (22% of normal). The F_1 of uncD478 had unusually high Ca^{2+}-dependent ATPase activity: K_m values for the Ca^{2+} ATPase of uncD478 and the wild type were 3.3 and 0.58 mM, respectively. It is not easy to draw any definite conclusions about the function of β from analysis of these mutants; before any conclusions are possible, more analyses and possibly more mutants are needed. However, results on uncD412, uncD11, and uncD478 suggest that β may have a site for divalent cations, in agreement with the finding that Mg^{2+} protected F_1 from inhibition by DCCD. Mitochondrial F_1's were shown to have tight binding sites for Mg (2 mol/mol) (213), a structural Mg site, and a second Mg site possibly involved in catalysis (209). EF_1 also has tight Mg (2 mol/mol) and Fe (1 to 2 mol/mol) (213). Both F_1's require free Mg^{2+} or Ca^{2+} in the assay mixture, possibly for the formation of Mg (or Ca) ATP as a substrate. Thus F_1's from the above mutants, including KF11, may have an altered Mg (or Ca) site(s) of the Mg (or Ca) ATP complex or an altered tight Mg^{2+} site involved in the catalysis.

Downie et al. (44) concluded that the uncG

gene codes for the γ subunit from studies using two plasmids carrying separate HindIII fragments, one carrying the promoter-proximal portion (uncBFEA genes) and the other carrying uncDC genes. These plasmids could not complement uncG mutations and no γ subunit was formed in in vitro experiments on protein synthesis. However, a plasmid carrying both HindIII fragments (uncBGEADC) could complement the uncG mutation and could be a template for the γ subunit. These results suggest that the uncG gene coding for γ may be located between uncA and uncD. Bragg et al. (15) reported a mutant in which γ had a molecular weight a few thousand less than normal. Recently, Kanazawa et al. (120) isolated four mutants and mapped the mutations within the γ subunit gene. KF10, KF1, and KF12 and KF13 were shown to have mutations within amino acid residues 1 to 82, 83 to 167, and 168 to 287, respectively. KF12 and KF13 have inactive F_1 with a structure similar to that of the wild type, although the complexes were easily dissociated during their purification. On the other hand, KF10 and KF1 had no inactive F_1 on the membrane, although the membrane was not permeable to protons. Kanazawa et al. also showed that the NR70 mutation could be mapped within residues 1 to 82. This strain was originally isolated by Rosen and is known to have no F_1 on the membrane and to have proton-permeable membranes (182). From these results, Kanazawa et al. (120) suggested that mutations in the amino-terminal half of the molecule (residues 1 to 167) resulted in more extensive defects in the assembly of F_1 than in alteration in the carboxyl half of the molecule (residue 168 to 287).

Primary Structures of δ and ϵ Subunits

Subunits δ and ϵ have polarities similar to those of soluble proteins (Table 2). The primary structures of ϵ from E. coli (114, 190) and chloroplasts (130, 257) have been determined and found to show about 20% homology (Table 3). The secondary structure deduced by the method of Chou and Fassman (31) suggested that δ is a long helical molecule (138). The α-helical content of the δ was 61% of the total residues and the longest α helix was 50 residues (138). The high helical content is in good agreement with results of previous circular dichroism measurements (α-helix content, 55 to 70%) (Table 3) (223). This finding is also consistent with the long Stoke's radius of the purified subunit (223). These results suggest that the δ subunit may form a stalk connecting the $\alpha\beta\gamma$ complex with F_0 (Fig. 2A).

Properties of Isolated δ and ϵ Subunits

As discussed above, a complex of α, β, and γ subunits had ATPase activity, but was unable to bind to F_0. Complete F_1 could be reconstituted from the $\alpha\beta\gamma$ complex and δ and ϵ subunits, suggesting that the δ and ϵ subunits are required for binding of the $\alpha\beta\gamma$ complex to the F_0 portion (49, 222). Results on the thermophilic bacterium suggest that the γ subunit acts as a gate regulating the passage of protons (253). The δ and ϵ from TF_1 could bind directly to F_0, although passive permeability of protons through F_0 was not affected by the binding. However, addition of the γ subunit together with δ and ϵ blocked the permeability of F_0 to protons. From a series of experiments on modifications with sulfhydryl reagents, McCarty suggested that γ is involved in regulation of proton flow through F_0 (149, 150). However, similar experiments with subunits from E. coli could not reproduce results with TF_1 subunits (50). Furthermore, Khananshvili and Gromet-Elhanan suggested that β and γ subunits could not be an integral part of a proton gate of chromatophores, because β- and γ-less chromatophores showed normal proton uptake driven by light (125). The other subunits of F_1 (α, δ, or ϵ) may regulate passage of protons through F_0 in this organelle. This conclusion may be appropriate if the complex of α, δ, and ϵ subunits has essentially the same interaction with F_0 as intact F_1. These results suggest that regulation of the proton gate may differ in different organelles. It is also possible that subtle control of proton flow from F_0 to F_1 is not attributable to a single subunit.

Mutations of δ and ϵ Subunits

Identification of δ mutants became easier with the use of complementation analysis with recombinant plasmids carrying defined portions of the unc DNA. Humbert et al. identified three independent mutants with similar properties (103). One of these mutants (uncH239) had approximately 70% as much ATPase activity as the wild type, and about half of the enzyme was associated with the membrane. The mutant F_1 was released even by washing with buffer of high ionic strength, whereas no release of F_1 was observed on washing wild-type membranes with this buffer. This observation is in agreement with the biochemical finding that the δ subunit is required for the binding of the $\alpha\beta\gamma$ complex to F_0. The mutation seems to affect the properties of F_0 and possibly its assembly, and membranes did not become leaky to protons after removal of F_1. Furthermore, ATP-driven formation of $\Delta\bar{\mu}_{H^+}$ could not be observed with washed mutant membranes bound with wild-type F_1. Noumi and Kanazawa independently isolated two mutants (KF5 and KF22) defective in the δ subunit (162). The membranes of the two strains showed different properties from those of uncH239. The membranes of KF5 and KF22 contained essen-

tially the same amounts of F_1 (estimated from the amounts of α and β) as that of the wild type, but these F_1's could not be released under conditions that removed all of the F_1 from wild-type membranes. They concluded that the F_1 had an abnormal assembly, because it had not ATPase activity. The membranes did not become permeable to protons even after extensive washing with buffer of low ionic strength. Thus, the assembly of F_1 and F_0 was changed appreciably by the mutation. It would be extremely interesting to know the structure of F_0 in uncH239 and KF5 or KF22. A mutant of ε (uncC) had a phenotype similar to those of uncB mutants with defective F_0 and was originally thought to be an F_0 mutant (33, 81). These results suggest that the assembly properties of F_0F_1 are changed by a mutation in uncC.

F_0, PROTON PATHWAY OF THE ATPase

Preparation of F_0

The entire F_1F_0 complex has been purified from mitochondria (3, 188, 215), chloroplasts (178), and bacteria (61, 66, 200, 219), and the subunit composition and function of F_0 have been studied. Here preparations of F_0F_1 or F_0 are discussed only briefly because they are discussed in detail in the excellent article by Fillingame (57). Sone et al. obtained F_0F_1 from thermophilic bacterium PS3 and reconstituted purified F_0F_1 into phospholipid vesicles (219). The F_0 part of their preparation consisted of three polypeptides of 19,000, 13,500, and 5,400 daltons. Sone et al. suggested that the highest-molecular-weight polypeptide may be unnecessary, because an F_0 preparation without this polypeptide showed about half the normal rate in proton conduction (220). It is interesting in this regard that F_0F_1 from S. faecalis does not have a subunit equivalent to the above peptide (1, 132). Foster and Fillingame developed a procedure for obtaining a pure preparation of F_0F_1 from E. coli (61). Their preparation contained three F_0 polypeptides of 24,000, 19,000, and 8,000 daltons, as determined by electrophoresis. Liposomes containing only the F_0 portion showed proton translocations utilizing a K^+ diffusion potential. In the DNA sequence shown below, these three polypeptides are indicated as F_0 subunits. These three components have been called a, b, and c subunits (65) or χ, ψ, and Ω subunits (58) (Table 1). We call them a, b, and c subunits because this terminology has been used more frequently. The F_0F_1's from S. faecalis (1, 132) and Mycobacterium phlei (22) also have three F_0 components. Friedl et al. prepared an ATPase complex of E. coli by a different procedure (66). Their preparation was equivalent in purity and activity (after reconstitution) to the

above preparation, although it was contaminated with small amounts of other polypeptides. Paradies et al. (171) measured the physical parameters of a comparable preparation and obtained an average value of 448,000 ± 23,000 for the molecular weight of E. coli F_0F_1 by small-angle X ray and laser light scattering. They also observed formation of a dimer of 930,000 daltons at 20°C. F_0F_1 of E. coli has also been prepared by other groups, but has not yet been characterized in detail (17, 184). F_0F_1 has been prepared from R. rubrum (9, 201), Clostridium pasteurianum (32), and Micrococcus luteus (199), although most preparations were not as well characterized as those from the thermophilic bacterium and E. coli. The F_0 portion has been purified from E. coli (69, 157) and from thermophilic bacterium PS3 (165) in reconstitutively active forms.

Structures and Functions of F_0 Subunits

The stoichiometry of F_0 subunits is still controversial: $a_1b_2c_{3-6}$ for thermophilic F_0 (109); $a_1b_2c_{10}$ for E. coli F_0 according to Foster and Fillingame (62) and $a_1b_2c_{12-15}$ for E. coli F_0 according to von Meyenburg et al. (238). The method applied for determining the value for the E. coli preparation, i.e., counting bands excised from polyacrylamide gel, is still open to criticism, as discussed by Senior and Wise (214). The calculation is based on the assumption that each band is recovered quantitatively. Presence of a dimer of b subunit in F_0 was also suggested by the formation of b-b cross-linking with azidonitrophenyl fluoride (203). As indicated above, all available evidence suggests that the c subunit functions as an oligomer, although different numbers of monomers have been found in the complex in different laboratories. Genetic evidence also suggests that the c subunit functions as an oligomer. Friedl et al. (67) showed that a diploid strain carrying the wild-type allele of the c subunit on the chromosome and the mutant allele on the F' plasmid had the mutant phenotype. Similarly, we could not complement a mutant of the c subunit with transducing phage λ asn5 carrying genes for F_0F_1, including the wild-type c subunit (226). These results suggest that oligomers of c subunits were formed by the mutant (coded by the E. coli chromosome) and wild type (coded by the F' plasmid or transducing phage) and that they showed a defective function as a proton channel. However, the mutation could be overcome with a high-copy-number plasmid carrying a gene for the wild-type c subunit (226), possibly because the oligomers had higher copies of the wild-type subunit than of that of the mutant.

Subunit c is also called DCCD-binding protein or proteolipid, because it binds DCCD and is

extremely hydrophobic, as judged from its solubility in chloroform-methanol. Sebald and Hoppe studied the protein extensively and reviewed their results recently (204). They determined the amino acid sequence of the c subunit from nine organisms (203). The sequence of the c subunit of $E.$ $coli$ determined from DNA (77, 88, 118) was completely consistent with their protein chemical results. All of the c subunits have two hydrophobic sequences connected by a central polar sequence. DCCD binds to a Glu residue in the center of the second hydrophobic sequence from the amino terminus in all c subunits except that of $E.$ $coli$, in which it binds to Asp of a homologous position (residue 61) (98, 204, 205, 207). Binding of DCCD to this Glu or Asp residue (204, 205) or genetic alteration of the Asp residue changes the proton permeability of F_0, suggesting that this residue is functional in proton translocation. Consistent with this possibility, the pH profile of the rate of H^+ translocation through thermophilic F_0 indicated a monoprotic H^+-binding site with a pK_a of 6.8 (165).

Sebald and co-workers constructed a "hairpin" model of the subunit after calculation of the secondary structure by four different prediction procedures (196, 204) and measurement of the circular dichroism spectrum (98, 204) in various conditions (Table 2; Fig. 2F). According to this model, two hydrophobic segments, possibly α helices, traverse the membrane in an antiparallel fashion and the central polar sequence is exposed to the cytoplasm. They speculated that the amino and carboxyl ends are on the opposite side of the membrane from F_1 and thus may not have specific functions, because these two sequences are both highly variable and vary in length in different species. This is consistent with the conclusion of Schneider et al. (202) from chemical modification studies that the amino terminus of the c subunit in $E.$ $coli$ membranes is oriented toward the medium. Senior and Wise (214) independently constructed an essentially similar model. Genetic evidence supports the hairpin model, as discussed below. Although the central hydrophobic portion of the c subunit is exposed to the F_1 side in the hairpin model, this subunit was only slightly affected by various proteases tested (93). This result suggests that the conformation of the hydrophilic portion may be resistant to proteases. Sebald and Hoppe (204) suggested that this segment, including a Pro residue, probably forms a β-turn which is known to be resistant to proteases. Immunochemical evidence also suggests that a portion of subunit c is exposed to the cytoplasmic surface of membranes of $E.$ $coli$. Inverted membrane vesicles of this bacterium became leaky to protons upon removal of F_1, because F_0 without F_1 functions as a passive proton path-

way. Loo and Bragg (134) showed that this leakage was prevented by antiserum to subunit c, suggesting that the antibodies bound to the hydrophilic portion of the c subunit and changed the entire F_0 conformation to a proton-impermeable form. Subunit c of chloroplasts (158) or yeast mitochondria (37) was shown to translocate H^+ when it was reconstituted into liposomes. However, the c subunit of $E.$ $coli$ (37) or the thermophilic bacterium did not translocate H^+ (219). Furthermore, a genetic study of $E.$ $coli$ indicated that membranes containing only the c subunit could not translocate protons (68). Recently, Shindler and Nelson observed H^+ conduction through black membranes containing only the c subunit from yeast, but found that the rate of conduction was low compared with that in native membranes (198).

Subunit b is a hydrophilic protein with a content of polar residues (48% polarity) similar to that in soluble proteins (Table 1). However, this protein has a hydrophobic stretch of 23 residues near the amino terminus, suggesting that it is embedded in the membrane through these residues (117) (Fig. 2E). This protein remains tightly bound to the membrane after solubilization of F_1 with dilute buffer (72) and is also difficult to solubilize with chaotropic agents (69). Furthermore, its predicted secondary structure has a high α-helical content (74, 196). Therefore, the amino-terminal domain of subunit b may be embedded and the other hydrophilic helical domain may be extruded from the membranes (117, 245) (Fig. 2E). Consistent with this model, subunit b is very sensitive to proteases: trypsin, subtilisin, or protease V8 digested this subunit to small polypeptides, and no intact subunit b was detectable after incubation at an enzyme/membrane ratio of 1/100 (93). Since protease treatment had no effect on H^+ permeability of the F_0 portion, the portion of the subunit extruding from the membrane does not form the proton pathway. Chymotrypsin cleaved about 25 to 30 residues from the carboxyl terminus and gave a defined cleavage product of 15,000, indicating that residues near the carboxyl terminus are not embedded in the membrane. This cleavage was prevented by F_1, suggesting that the chymotrypsin site is near the F_1 binding site. F_1 could bind to the protease-treated membranes, although when rebound to protease-treated membranes, even chymotrypsin-treated membranes, it was insensitive to DCCD and showed no ATP-driven proton conduction. These results suggest that subunit b is required for correct binding of F_1. Similar but somewhat different results were reported for thermophilic and mitochondrial F_0's (173, 220). Digestion of a 13,000-dalton protein of thermophilic F_0, possibly equivalent to subunit b of $E.$ $coli$, had no effect on proton conduc-

tion of F_0, but thermophilic F_0 lost activity to bind F_1 after this treatment. Pedersen et al. (173) treated F_1-depleted submitochondrial vesicles with trypsin and found that after treatment they could still bind F_1. The rebound F_1 was still sensitive to DCCD, but did not show ATP-driven H^+ translocation. Thus, the interaction of F_1 with the b subunit may differ in different species. The topology of the b subunit was recently studied by Hoppe et al. with the hydrophobic photoreactive agent 1-palmitoyl, 2-(2-azido-4-nitro)benzoyl sn-glycero[^{3}H]phosphocholine (94). Subunit b was labeled, but essentially no radioactivity was found in other subunits. By sequencing the labeled protein, they found that Cys-21 was predominantly labeled, although there was some label at Asn-2 and Trp-26. As the nitrophenyl azide group becomes attached to the polar head group of phospholipid, the modified residues were suggested to be close to the surface of the lipid bilayer. The same Cys-21 residue was labeled predominantly with the hydrophobic photoreactive reagents iodonaphtylazide and trifluoro-methyl-iodophenyl-diazirine (203), suggesting that this residue is in a hydrophobic environment near the polar surface of the bilayer. Cys-21 is actually within the hydrophobic stretch in the amino-terminal portion (residues 11 to 33) which was suggested to be embedded in the membranes (74, 117).

Subunit a has a typical amino acid sequence for a membrane protein with a low content of polar amino acid residues (Table 2). The secondary structure of this subunit has been predicted (74, 196). The protein has long stretches of hydrophobic residues and short hydrophilic segments. Seven hydrophobic segments of 15 to 30 residues (60, 196) were predicted, which possibly span the membrane (Fig. 2D). The amino-terminal portion (40 residues) is hydrophilic and could extrude from the membranes (196). This protein has homology to subunit 6 of yeast mitochondria (117), suggesting that subunits a and 6 are equivalent F_0 components (Table 3). However, there is less homology between subunits a and 6 of human (6) or mouse (11) mitochondria (117) (Table 3). Yeast subunit 6 carries two oligomycin-resistant loci, $oli-2$ and $oli-4$ (214). Senior and Wise (214) pointed out that the amino acid sequences around these two loci are conserved in human and mouse subunit 6, whereas in $E.\ coli$ subunit a only the region around $oli-2$ is conserved. Thus, $E.\ coli$ subunit a does not have a complete oligomycin site, which is consistent with the finding that oligomycin does not inhibit the $E.\ coli$ enzyme. Hoppe et al. (93) showed that trypsin and chymotrypsin were not accessible to the corresponding sites, and only 15 to 20% of the mole-

cule was cleaved by nonspecific subtilisin. It is of interest that subtilisin at an enzyme/membrane protein ratio of 1/100 caused almost complete disappearance of all membrane proteins of molecular weights higher than 15,000 except subunit a. Hoppe et al. speculated that hydrophilic sequences near the amino terminus were cleaved by the enzyme, but that other portions were not accessible to the enzyme because they were embedded in the phospholipid bilayer. A model of F_0F_1 including predicted conformation of a, b, and c subunits is shown in Fig. 4.

Mutations of F_0 Subunits

Mutants of subunit c have been characterized the most extensively, and altered amino acid residues have been mapped in the primary structure. Sebald and co-workers found that Asp at position 61 is replaced by Asn (95) or Gly (97, 239) in subunit c of proton-impermeable mutants. Thus, the Asp residue at this position seems to be essential for proton permeability. As discussed above, this residue is in a second hydrophobic cluster and is conserved as Glu in the sequence of all other organisms so far examined. Furthermore, DCCD binds to this residue and abolishes the proton conductance of F_0 (204, 205). These findings suggest that a carboxyl group at this position is essential for H^+ conduction. Sebald and co-workers also examined amino acid substitutions in six DCCD-resistant mutants (95, 204). These mutants had F_0 with normal proton permeability but were resistant to DCCD. In these mutants, Ile at position 28 was replaced by Val or Thr. The Val substitution gave moderate DCCD resistance, but Thr gave high resistance. It is noteworthy that a mutation at position 28 changed the property of the residue at 61, making it inaccessible to DCCD. Sebald and Hoppe proposed that residue 28 is near residue 61 and that residues 28 and 61 may form a binding site for DCCD (204). Their proposal is consistent with the hairpin model of subunit c discussed above. Gibson also identified altered residues in $uncE$ mutants: in $uncE410$, Leu at position 31 was replaced by Phe, and in $uncE408$, Pro at 64 was replaced by Leu (80). He showed that $uncE410$ lacked proton conduction and that the c subunit of $uncE408$ was partially defective. Thus, the residues at 64 and 31 were closely related to proton conduction. These results are also consistent with the hairpin model of subunit c.

Sebald and co-workers identified altered residues of oligomycin-resistant cells of $N.\ crassa$ (204, 206) and $Saccharomyces\ cerevisiae$ (204, 240). Their results indicated that for oligomycin sensitivity of ATPase three proteins, subunit c, subunit 6, and oligomycin sensitivity-conferring protein, are required. It is interesting that the

altered residues were mapped in the carboxy half of subunit c. The residues altered in oligomycin-resistant mutants may be the binding site for oligomycin, although this binding site has not yet been determined.

The $uncB402$ mutant is the first strain $E.\ coli$ found to be defective in the F_0 portion (27). Kanazawa et al. mapped this gene on a defined DNA segment that differed from that of the gene for subunit c (118). Downie et al. concluded that $uncB$ codes for the a subunit (42). They analyzed peptides synthesized in vitro, using a series of deletion plasmids, and found that only subunit a could not be synthesized by pAN95 with a deletion in the $uncB$ region. The gene-polypeptide relationship was finally established by the results of DNA sequencing (77, 117, 161). The $uncB402$ mutant had negligible activity for ATP synthesis, although it had normal F_1 bound to the membrane (27, 35). The F_1 of this mutant was dissociated from F_0 more readily than that of the wild type (59). The ATPase activity of mutant F_1 was not sensitive to inhibition by DCCD (35, 90), although this reagent reacts covalently with subunit c (59). These findings indicate that the interaction between F_1 and F_0 was altered drastically by the $uncB$ mutation, possibly due to an altered assembly of F_0 subunits. The F_0 of the mutant shows <5% of the H^+ conduction of the wild-type F_0, as demonstrated by measuring H^+ conduction through crude membranes (59, 90) or liposomes (59) containing partially purified F_0. Fillingame et al. (59) cloned genes of $uncB402$ into the λ transducing phage ($\lambda\ uncB402$) and showed that all subunits of F_0F_1 except the a subunit could be synthesized in vitro by using DNA from the transducing phage. They found a protein of 18,000 daltons with $\lambda\ uncB402$ DNA but not wild-type DNA as template. The wild-type a subunit migrated to the position of 24,000 molecular weight in gel electrophoresis, although the actual molecular weight is higher than this value (Table 2). These results are consistent with the finding that $uncB402$ is amber suppressible. It is premature to discuss the function of the a subunit from results on one mutant, but results on the mutant suggest the importance of the subunit in proton conduction and F_1 binding.

Downie et al. showed that $uncF$ coded for subunit b (42). Their $uncF469$ and $uncF476$ mutants had high ATPase activity in the cytoplasm and F_0's that were impermeable to protons, suggesting that subunit b is related to binding of F_1 and the proton pathway. Friedl et al. (65) isolated polar mutant AS12, using Mu phage. Membranes of this strain contained intact a and c subunits as subunits of F_0. Proton conduction of these membranes was greatly lowered, suggesting that the b subunit is necessary for efficient proton conduction. Noumi and Kanazawa (162) recently isolated a mutant (KF8) of subunit b with different properties from those of the above strains. Their mutant is defective in oxidative phosphorylation and thus is unable to grow on succinate like other unc mutants. The membrane of KF8 has normal activity of H^+ channels, as shown by quenching of a fluorescent dye. The ATPase activity of the membrane is low, but wild-type F_1 can bind to the membrane and drive ATP-driven proton translocation. The F_0F_1 of KF8 does not seem to be completely reversible and is only defective in synthesis of ATP coupled to H^+ conduction. Taking the results of these three groups together, we may conclude that the b subunit is not itself the catalytic component of the proton pathway but is important in forming the active H^+ channel.

The roles of the three F_0 subunits in formation of F_0 were studied extensively by Friedl et al. (68). They constructed strains by combining a deletion mutant ($\Delta\ uncIBEFHA$) and recombinant plasmids and established strains in which only one or two types of subunits were synthesized and incorporated into F_0. From analyses of these strains, they concluded that all three subunits are required for efficient formation of the H^+ channel. Membranes containing only subunit a or b bound F_1, but membranes containing only subunit c could not bind F_1. Membranes with the a and b subunits bound slightly more F_1 than the sum of the amounts bound by membranes with the a or b subunit, respectively. Thus, the proton channel of F_0 is formed by the close interactions of the three subunits, and the F_1 binding site is formed from a and b subunits.

SUMMARY

We have tried to summarize current information on the structure and function of bacterial proton-translocating ATPase (F_0F_1), mainly obtained on F_0F_1 of $E.\ coli$. Ten years ago, little attention was paid to the bacterial enzyme by investigators who were interested in the synthesis of ATP. However, interest in bacteria increased once it was established that the proton-translocating ATPases of energy-transducing membranes of bacteria, animals, and plants are similar in structure and function. The F_0F_1's from the thermophilic bacterium PS3 and $E.\ coli$ were highly purified and could be reconstituted into liposomes, and these liposomes were capable of coupling $\Delta\bar{\mu}_{H^+}$ with hydrolysis or synthesis of ATP. All five subunits of F_1 have been obtained from the two bacteria, and functional F_1 has been reconstituted from these subunits. Hybrid enzymes were obtained by combinations

of subunits from the two bacteria, suggesting functional and structural homologies of the enzymes of these distantly related bacteria.

A great advantage of using *E. coli* is that genetic techniques can be used. As discussed extensively above, mutants of each subunit were isolated and the gene cluster for the enzyme was defined. This gene cluster was subsequently cloned into λ phages and plasmids, and its DNA sequence was determined. The primary structures of all of the subunits (a, b, c, α, β, γ, δ, ε) of the *E. coli* enzyme were determined, and higher-ordered structures of the subunits were deduced. Comparison of the primary structures of the β subunits of *E. coli* and chloroplasts (also determined from the DNA sequences) or mitochondria (determined by protein chemistry) revealed high degrees of homology. The primary sequences of the ε subunit and the a and c subunits of chloroplasts were also determined and found to show less homology with those of *E. coli* subunits. The genes coded by DNA of chloroplasts or mitochondria have been sequenced. Cloning of the nuclear genes for F_0F_1 of eucaryotic cells has been started, and the nuclear gene for the c subunit of *Neurospora* sp. has already been reported. The α and β subunits both have binding sites for nucleotides as shown by chemical modification and studies on isolated subunits. The primary structures of α and β were compared with those of enzymes for which the substrate or effector is ATP or ADP. Homologous sequences were found in these proteins and were suggested to be good subjects for future studies on nucleotide binding sites. Biochemical studies, including experiments using photoaffinity label, suggested that β or the interface of α and β may be the catalytic site, although this is still uncertain. A conceptual model of F_0F_1 of *E. coli* was drawn based on biochemical and molecular biological studies discussed in this review (Fig. 4). Results of predicted secondary structures, subunit stoichiometry, and subunit interactions were included in this model.

Mutants of the enzyme have been useful in determining the gene-subunit relationship and the roles of the subunits. As discussed above, it is now possible to identify altered residues in mutants of the c subunit and to map mutations of the γ subunit in the primary structure of the subunit. From more detailed studies on this subject it should be possible to identify functional residues in the active site and proton pathway. Through experiments on chemical modifications it has become possible to identify modified residues definitely instead of speculating on these modifications from results with reagents of known specificities. From combined biochemical and genetic studies it may be possible to clarify the mechanism of ATP synthesis coupled to $\Delta \bar{\mu}_{H^+}$ at a molecular level.

The mechanism of ATP synthesis in F_1 has been studied extensively, using the beef heart enzyme, as discussed briefly above. The present data support the "binding change mechanism," rather than a mechanism in which H^+ (transported through F_0) directly participates in catalysis. Although few studies on this subject have been carried out with the *E. coli* enzyme, further detailed studies with bacterial enzymes may be fruitful. Isolated subunits may give information on partial reactions, including conformational change. We may be able to form hybrid enzymes from mutant and wild-type subunits, as such experiments were possible with F_1 from different bacterial species. Kinetic analyses with such hybrid enzymes may be useful in understanding the mechanism of the enzyme. Studies on the biogenesis and assembly of this complex enzyme have become interesting. In this article, we discussed one simple question, "What is the mechanism regulating synthesis of the complex of stoichiometric amounts of different subunits?" This question is still difficult to answer conclusively. More refined experiments on the regulatory system will be carried out in the near future.

We hope that our effort to review both biochemical and molecular biological approaches to this subject will stimulate further progress in this field.

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